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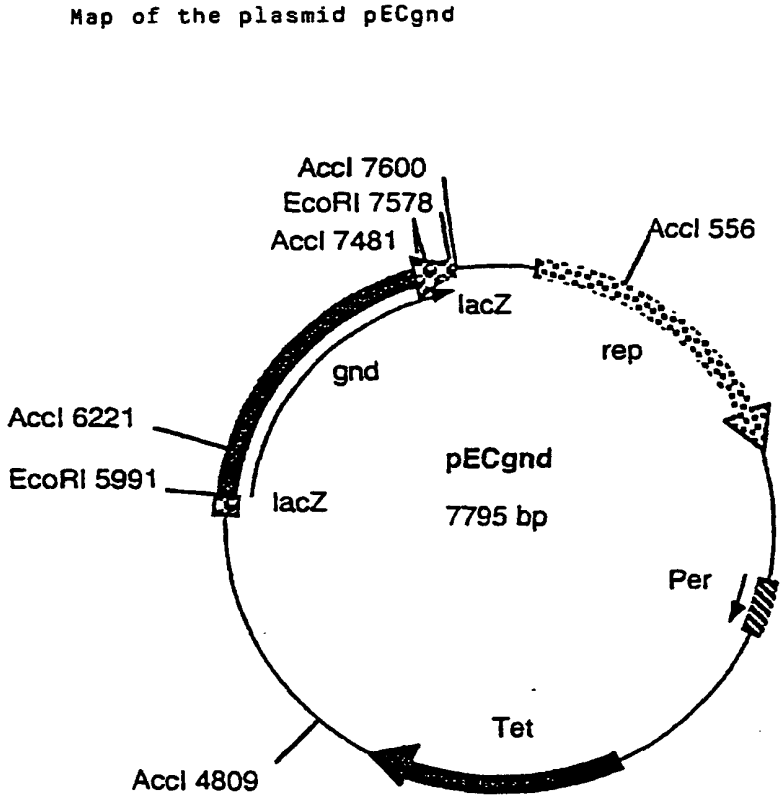
(54) Title: PROCESS FOR THE FERMENTATIVE PREPARATION OF L-AMINO ACIDS WITH AMPLIFICATION OF THE GND GENE



(57) Abstract: The invention relates to a process for the preparation of L-amino acids by fermentation of coryneform bacteria, which comprises carrying out the following steps: a) fermentation of the desired L-amino acid-producing bacteria in which at least the gnd gene is amplified, b) concentration of the L-amino acid in the medium or in the cells of the bacteria and c) isolation of the L-amino acid produced.

WO 01/71012 A1

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Process for the fermentative preparation of L-amino acids with amplification of the gnd gene

The invention relates to a process for the fermentative preparation of L-amino acids, in particular L-lysine, L-threonine, L-isoleucine and L-tryptophan, using coryneform bacteria in which at least the gnd gene is amplified.

Prior art

L-Amino acids are used in animal nutrition, in human medicine and in the pharmaceuticals industry.

It is known that amino acids are prepared by fermentation from strains of coryneform bacteria, in particular Corynebacterium glutamicum. Because of their great importance, work is constantly being undertaken to improve the preparation processes. Improvements to the process can relate to fermentation measures, such as e.g. stirring and supply of oxygen, or the composition of the nutrient media, such as e.g. the sugar concentration during the fermentation, or the working up to the product form by e.g. ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites, such as e.g. the threonine analogue α-amino-β-hydroxyvaleric acid (AHV), or are auxotrophic for metabolites of regulatory importance and produce L-amino acids such as e.g. threonine are obtained in this manner.

Methods of the recombinant DNA technique have also been employed for some years for improving the strain of Corynebacterium glutamicum strains which produce L-amino acids.

Object of the invention

The inventors had the object of providing improved processes for the fermentative preparation of L-amino acids with coryneform bacteria.

Description of the invention

L-Amino acids are used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and especially in animal nutrition. There is therefore a general interest in providing new improved processes for the preparation of amino acids.

The invention provides a process for the fermentative preparation of L-amino acids, in particular L-lysine, L-threonine, L-isoleucine and L-tryptophan, using coryneform bacteria in which the nucleotide sequence which codes for the enzyme 6-phosphogluconate dehydrogenase (EC number 1.1.1.44) (gnd gene) is amplified, in particular over-expressed.

The strains employed preferably already produce L-amino acids before amplification of the gnd gene.

Preferred embodiments are to be found in the claims.

The term "amplification" in this connection describes the increase in the intracellular activity of one or more enzymes in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or genes, using a potent promoter or using a gene which codes for a corresponding enzyme having a high activity, and optionally combining these measures.

The microorganisms which the present invention provides can prepare L-amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. They are representatives of

coryneform bacteria, in particular of the genus Corynebacterium. Of the genus Corynebacterium, there may be mentioned in particular the species Corynebacterium glutamicum, which is known among experts for its ability to produce L-amino acids.

Suitable strains of the genus Corynebacterium, in particular of the species Corynebacterium glutamicum, are, for example, the known wild-type strains

Corynebacterium glutamicum ATCC13032
Corynebacterium acetoglutamicum ATCC15806
Corynebacterium acetoacidophilum ATCC13870
Corynebacterium thermoaminogenes FERM BP-1539
Brevibacterium flavum ATCC14067
Brevibacterium lactofermentum ATCC13869
Brevibacterium divaricatum ATCC14020

and L-amino acid-producing mutants prepared therefrom,

such as, for example, the L-threonine-producing strains

Corynebacterium glutamicum ATCC21649
Brevibacterium flavum BB69
Brevibacterium flavum DSM5399
Brevibacterium lactofermentum FERM-BP 269
Brevibacterium lactofermentum TBB-10

and such as, for example, the L-isoleucine-producing strains

Corynebacterium glutamicum ATCC 14309
Corynebacterium glutamicum ATCC 14310
Corynebacterium glutamicum ATCC 14311
Corynebacterium glutamicum ATCC 15168
Corynebacterium ammoniagenes ATCC 6871

and such as, for example, the L-tryptophan-producing strains

Corynebacterium glutamicum ATCC21850
Corynebacterium glutamicum KY9218(pKW9901)

and such as, for example, the L-lysine-producing strains
Corynebacterium glutamicum FERM-P 1709
Brevibacterium flavum FERM-P 1708
Brevibacterium lactofermentum FERM-P 1712
Corynebacterium glutamicum FERM-P 6463
Corynebacterium glutamicum FERM-P 6464
Corynebacterium glutamicum DSM5715
Corynebacterium glutamicum DM58-1
Corynebacterium glutamicum DSM12866.

It has been found that coryneform bacteria produce L-amino acids, in particular L-lysine, L-threonine, L-isoleucine and L-tryptophan, in an improved manner after over-expression of the gnd gene which codes for 6-phosphogluconate dehydrogenase (EC number 1.1.1.44).

The gnd gene codes for the enzyme 6-phosphogluconate dehydrogenase , which catalyses the oxidative decarboxylation of 6-phosphogluconic acid to ribulose 5-phosphate. The nucleotide sequence of the gnd gene is disclosed in JP-A-9-224662. The gnd gene described in the text reference mentioned is used according to the invention for the first time. Alleles of the gnd gene which result from the degeneracy of the genetic code or due to sense mutations of neutral function can furthermore be used.

To achieve an amplification (e.g. over-expression), the number of copies of the corresponding genes is increased, or the promoter and regulation region or the ribosome binding site upstream of the structural gene is mutated. Expression cassettes which are incorporated upstream of the structural gene act in the same way. By inducible promoters, it is additionally possible to increase the expression in the course of fermentative L-amino acid formation. The expression is likewise improved by measures to prolong the life of the m-RNA. Furthermore, the enzyme activity is also increased by preventing the degradation of the enzyme protein. The genes or gene constructs are either

present here in plasmids with a varying number of copies, or are integrated and amplified in the chromosome. Alternatively, an over-expression of the genes in question can furthermore be achieved by changing the composition of the media and the culture procedure.

Instructions in this context can be found by the expert, inter alia, in Martin et al. (Bio/Technology 5, 137-146 (1987)), in Guerrero et al. (Gene 138, 35-41 (1994)), Tsuchiya and Morinaga (Bio/Technology 6, 428-430 (1988)), in Eikmanns et al. (Gene 102, 93-98 (1991)), in European Patent Specification EPS 0 472 869, in US Patent 4,601,893, in Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991), in Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)), in LaBarre et al. (Journal of Bacteriology 175, 1001-1007 (1993)), in Patent Application WO 96/15246, in Malumbres et al. (Gene 134, 15-24 (1993)), in Japanese Laid-Open Specification JP-A-10-229891, in Jensen and Hammer (Biotechnology and Bioengineering 58, 191-195 (1998)) and in known textbooks of genetics and molecular biology.

By way of example, 6-phosphogluconate dehydrogenase was over-expressed with the aid of a plasmid. The E. coli - C. glutamicum shuttle vector pEC-T18mob2 shown in Figure 1 was used for this. After incorporation of the gnd gene into the EcoRI cleavage site of pEC-T18mob2, the plasmid pECgnd shown in Figure 2 was formed.

Other plasmid vectors which are capable of replication in C. glutamicum, such as e.g. pEKEx1 (Eikmanns et al., Gene 102:93-98 (1991)) or pZ8-1 (EP-B- 0 375 889), can be used in the same way.

In addition, it may be advantageous for the production of L-amino acids to amplify one or more enzymes of the particular biosynthesis pathway, of glycolysis, of anaplerosis, of the pentose phosphate pathway or of amino

acid export, in addition to amplification of the gnd gene which codes for 6-phosphogluconate dehydrogenase.

Thus, for example, in particular for the preparation of L-threonine, one or more genes chosen from the group consisting of

- the hom gene which codes for homoserine dehydrogenase (Peoples et al., Molecular Microbiology 2, 63-72 (1988)) or the hom^{dr} allele which codes for a "feed back resistant" homoserine dehydrogenase (Archer et al., Gene 107, 53-59 (1991),
- the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns et al., Journal of Bacteriology 174: 6076-6086 (1992)),
- the pyc gene which codes for pyruvate carboxylase (Peters-Wendisch et al., Microbiology 144: 915-927 (1998)),
- the mqo gene which codes for malate:quinone oxidoreductase (Molenaar et al., European Journal of Biochemistry 254, 395-403 (1998)),
- the tkt gene which codes for transketolase (accession number AB023377 of the databank of European Molecular Biology Laboratories (EMBL, Heidelberg, Germany)),
- the zwf gene which codes for glucose 6-phosphate dehydrogenase (JP-A-09224661),
- the thrE gene which codes for threonine export (DE 199 41 478.5; DSM 12840),
- the zwa1 gene (DE 199 59 328.0; DSM 13115),
- the eno gene which codes for enolase (DE: 199 41 478.5)

can be amplified, in particular over-expressed, at the same time.

Thus, for example, in particular for the preparation of L-lysine, one or more genes chosen from the group consisting of

- the dapA gene which codes for dihydrodipicolinate synthase (EP-B 0 197 335),
- a lysC gene which codes for a feed back resistant aspartate kinase (Kalinowski et al. (1990), Molecular and General Genetics 224: 317-324),
- the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the pyc gene which codes for pyruvate carboxylase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the mqo gene which codes for malate-quinone oxidoreductase (Molenaar et al., European Journal of Biochemistry 254, 395-403 (1998)),
- the tkt gene which codes for transketolase (accession number AB023377 of the databank of European Molecular Biologies Laboratories (EMBL, Heidelberg, Germany)),
- the zwf gene which codes for glucose 6-phosphate dehydrogenase (JP-A-09224661),
- the lysE gene which codes for lysine export (DE-A-195 48 222),
- the zwa1 gene (DE 199 59 328.0; DSM 13115),
- the eno gene which codes for enolase (DE 199 47 791.4)

can be amplified, in particular over-expressed, at the same time.

It may furthermore be advantageous for the production of L-amino acids at the same time to attenuate one or more of the genes chosen from the group consisting of:

- the pck gene which codes for phosphoenol pyruvate carboxykinase (DE 199 50 409.1; DSM 13047),
- the pgi gene which codes for glucose 6-phosphate isomerase (US 09/396,478, DSM 12969),
- the poxB gene which codes for pyruvate oxidase (DE 199 51 975.7; DSM 13114),
- the zwa2 gene (DE: 199 59 327.2; DSM 13113)

in addition to the amplification of the gnd gene.

In addition to over-expression of 6-phosphogluconate dehydrogenase, it may furthermore be advantageous for the production of L-amino acids to eliminate undesirable side reactions (Nakayama: "Breeding of Amino Acid Producing Micro-organisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The microorganisms prepared according to the invention can be cultured continuously or discontinuously in the batch process (batch culture) or in the fed batch (feed process) or repeated fed batch process (repetitive feed process) for the purpose of L-amino acid production. A summary of known culture methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik [Bioprocess Technology 1. Introduction to Bioprocess Technology (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und

periphere Einrichtungen [Bioreactors and Peripheral Equipment] (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of the particular microorganisms in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981). Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, such as e.g. soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as e.g. palmitic acid, stearic acid and linoleic acid, alcohols, such as e.g. glycerol and ethanol, and organic acids, such as e.g. acetic acid, can be used as the source of carbon. These substances can be used individually or as a mixture. Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture. Potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as e.g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the above-mentioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH. Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, e.g. antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 20°C to 45°C, and preferably 25°C to 40°C. Culturing is continued until a maximum of L-amino acid has formed. This target is usually reached within 10 hours to 160 hours.

The analysis of L-amino acids can be carried out by anion exchange chromatography with subsequent ninhydrin derivatization, as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190), or it can take place by reversed phase HPLC as described by Lindroth et al. (Analytical Chemistry (1979) 51:. 1167-1174).

The following microorganism has been deposited at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty:

Escherichia coli K-12 DH5α/pEC-T18mob2 as DSM 13244

The following figures are attached:

- Figure 1: Map of the plasmid pEC-T18mob2
- Figure 2: Map of the plasmid pECgnd
- Figure 3: Map of the plasmid pBGNA
- Figure 4: Map of the plasmid pCR2.1poxBint

The base pair numbers stated are approx. values obtained in the context of reproducibility.

The abbreviations used have the following meaning:

Re Figure 1:

Tet:	Resistance gene for tetracycline
oriV:	Plasmid-coded replication origin of E. coli
RP4mob:	mob region for mobilizing the plasmid
rep:	Plasmid-coded replication origin from C. glutamicum plasmid pGA1
per:	Gene for controlling the number of copies from pGA1
lacZ-alpha:	lacZα gene fragment (N-terminus) of the β-Galactosidase gene

Re Figure 2:

Tet:	Resistance gene for tetracycline
rep:	Plasmid-coded replication origin from C. glutamicum plasmid pGA1
per:	Gene for controlling the number of copies from PGA1
lacZ	Cloning relict of the lacZα gene fragment from pEC-T18mob2
gnd:	6-Phosphogluconate dehydrogenase gene

Re Figure 3:

LacP:	Promoter of the E. coli lactose operon
CMV:	Promoter of cytomegalovirus
ColE1:	Replication origin of the plasmid ColE1

TkpolyA:	Polyadenylation site
Kan r:	Kanamycin resistance gene
SV40ori:	Replication origin of Simian virus 40
gnd:	6-Phosphogluconate dehydrogenase gene

Re Figure 4:

ColE1 ori:	Replication origin of the plasmid ColE1
lacZ:	Cloning relict of the lacZα gene fragment
f1 ori:	Replication origin of phage f1
KmR:	Kanamycin resistance
ApR:	Ampicillin resistance
poxBint:	internal fragment of the poxB gene

Moreover, the following abbreviations have been used:

AccI:	Cleavage site of the restriction enzyme AccI
BamHI:	Cleavage site of the restriction enzyme BamHI
EcoRI:	Cleavage site of the restriction enzyme EcoRI
HindIII:	Cleavage site of the restriction enzyme HindIII
KpnI:	Cleavage site of the restriction enzyme KpnI
PstI:	Cleavage site of the restriction enzyme PstI
PvuI:	Cleavage site of the restriction enzyme PvuI
SalI:	Cleavage site of the restriction enzyme SalI
SacI:	Cleavage site of the restriction enzyme SacI
SmaI:	Cleavage site of the restriction enzyme SmaI
SphI:	Cleavage site of the restriction enzyme SphI
XbaI:	Cleavage site of the restriction enzyme XbaI
XhoI:	Cleavage site of the restriction enzyme XhoI

Examples

The following examples will further illustrate this invention. The molecular biology techniques, e.g. plasmid DNA isolation, restriction enzyme treatment, ligations, standard transformations of Escherichia coli etc. used are, (unless stated otherwise), described by Sambrook et al., (Molecular Cloning. A Laboratory Manual (1989) Cold Spring Harbour Laboratories, USA).

Example 1

Construction of a gene library of Corynebacterium glutamicum strain AS019

A DNA library of Corynebacterium glutamicum strain AS019 (Yoshihama et al., Journal of Bacteriology 162, 591-597 (1985)) was constructed using ë Zap Express™ system, (Short et al., (1988) Nucleic Acids Research 16: 7583-7600), as described by O'Donohue (O'Donohue, M. (1997). The Cloning and Molecular Analysis of Four Common Aromatic Amino Acid Biosynthetic Genes from Corynebacterium glutamicum. Ph.D. Thesis, National University of Ireland, Galway). ë Zap Express™ kit was purchased from Stratagene (Stratagene, 11011 North Torrey Pines Rd., La Jolla, California 92037) and used according to the manufacturers instructions. AS019-DNA was digested with restriction enzyme Sau3A and ligated to BamHI treated and dephosphorylated ë Zap Express™ arms.

Example 2

Cloning and sequencing of the gnd gene

1. Construction of a gnd probe
A radiolabelled oligonucleotide, internal to the gnd gene, was used to probe the AS019 ë Zap Express™ library described above. The oligonucleotide was produced using degenerate PCR primers internal to the gnd gene. The

degenerate nucleotide primers designed for the PCR amplification of gnd DNA fragments were as follows:

gnd1: 5' ATG GTK CAC ACY GGY ATY GAR TA 3'
gnd2: 5' RGT CCA YTT RCC RGT RCC YTT 3'

with R=A+G; Y=C+T; K=T+G.

The estimated size of the resulting PCR product was 252 bp approximately.

Optimal PCR conditions were determined to be as follows:

35 cycles
94°C for 1 minute
55°C for 1 minute
72°C for 30 seconds
2.5 - 3.5 mM $MgCl_2$
100 - 150 ng AS019 genomic DNA

Sequence analysis of the resulting PCR product confirmed the product to be an internal portion of a gnd gene. Sequence analysis was carried out using the universal forward and reverse primers, and T7 sequencing kit from Pharmacia Biotech, (St. Albans, Herts, UK). The sequence of the PCR product is shown in SEQ ID No. 1.

2. Cloning
Screening of the AS019 ë Zap Express™ library was carried out according to the ë Zap Express™ system protocol, (Stratagene, 11011 North Torrey Pines Rd., La Jolla, California 92037). Southern Blot analysis was then carried out on isolated clones. Southern transfer of DNA was as described in the Schleicher and Schuell protocols manual employing Nytran™ as membrane („Nytran, Modified Nylon-66 Membrane Filters" (March 1987), Schleicher and Schuell, Dassel, Germany). Double stranded DNA fragments, generated using the same primers and optimal PCR conditions as described above, were radiolabelled with α-^{32}P-dCTP using

the Multiprime™ DNA labelling kit from Amersham Life Science (Amersham Pharmacia Biotech UK Limited, Little Chalfont, Buckinghamshire, UK) according to the manufacturers instructions. Prehybridisation, hybridization and washing conditions were as described in the Schleicher and Schuell protocols manual. Autoradiography was carried out according to the procedure outlined in the handbook of Sambrook et al. using AgFa Curix RPIL film. Thus several gnd clones were identified. Plasmid DNA was isolated from one of the clones, designated pBGNA (Figure 3) and chosen for further analysis.

3. Sequencing

The Sanger Dideoxy chain termination method of Sanger et al. (Proceedings of the National Academy of Sciences USA 74, 5463-5467 (1977)) was used to sequence the cloned insert of pBGNA. The method was applied using the T7 sequencing kit and α-^{35}S-dCTP from Pharmacia Biotech (St. Albans, Herts, UK). Samples were electrophoresed for 3-8 hours on 6% polyacrylamide/urea gels in TBE buffer at a constant current of 50 mA, according to the Pharmacia cloning and sequencing instructions manual („T7 Sequencing™ Kit",ref.XY-010-00-19, Pharmacia Biotech, 1994). Sequence analysis was carried out using internal primers designed from the sequence known of the internal gnd PCR product (SEQ ID NO 1) allowing the entire gnd gene sequence to be deduced.

The sequences of the internal primers were as follows:

Internal primer 1: 5' GGT GGA TGC TGA AAC CG 3'
Internal primer 2: 5' GCT GCA TGC CTG CTG CG 3'
Internal primer 3: 5' TTG TTG CTT ACG CAC AG 3'
Internal primer 4: 5' TCG TAG GAC TTT GCT GG 3'

Sequence obtained was then analyzed using the DNA Strider programme, (Marck (1988), Nucleic Acids Research 16: 1829-1836), version 1.0 on an Apple Macintosh computer. This

program allowed for analyses such as restriction site usage, open reading frame analysis and codon usage determination. Searches between DNA sequence obtained and those in EMBL and Genbank databases were achieved using the BLAST programme (Altschul et al., (1997), Nucleic Acids Research 25: 3389-3402). DNA and protein sequences were aligned using the Clustal V and Clustal W programs (Higgins and Sharp, 1988 Gene 73: 237-244).

The sequence thus obtained is shown in SEQ ID NO 2. The analysis of the nucleotide sequence obtained revealed an open reading frame of 1377 base pairs which was designated as gnd gene. It codes for a protein of 459 amino acids shown in SEQ ID NO 3.

Example 3

Preparation of the shuttle vector pEC-T18mob2

The E. coli - C. glutamicum shuttle vector pEC-T18mob2 was constructed according to the prior art.

The vector contains the replication region rep of the plasmid pGA1 including the replication effector per (US-A-5,175,108; Nesvera et al., Journal of Bacteriology 179, 1525-1532 (1997)), the tetracycline resistance-imparting tetA(Z) gene of the plasmid pAG1 (US-A- 5,158,891; gene library entry at the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) with accession number AF121000), the replication region oriV of the plasmid pMB1 (Sutcliffe, Cold Spring Harbor Symposium on Quantitative Biology 43, 77-90 (1979)), the lacZ gene fragment including the lac promoter and a multiple cloning site (mcs) (Norrander et al. Gene 26, 101-106 (1983)) and the mob region of the plasmid RP4 (Simon et al.,(1983) Bio/Technology 1:784-791).

The vector constructed was transformed in the E. coli strain DH5α (Hanahan, In: DNA cloning. A practical

approach. Vol. I. IRL-Press, Oxford, Washington DC, USA, 1985). Selection for plasmid-carrying cells was made by plating out the transformation batch on LB agar (Sambrook et al., Molecular cloning: a laboratory manual. 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., USA, 1989), which had been supplemented with 5 mg/l tetracycline. Plasmid DNA was isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction with the restriction enzyme EcoRI and HindIII subsequent agarose gel electrophoresis (0.8%).

The plasmid was called pEC-T18mob2 and is shown in Figure 1. It is deposited in the form of the strain Escherichia coli K-12 strain DH5α/pEC-T18mob2 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) as DSM 13244.

Example 4

Cloning of the gnd gene into the E. coli - C. glutamicum shuttle vector pEC-T18mob2

PCR was used to amplify DNA fragments containing the entire gnd gene of C. glutamicum and flanking upstream and downstream regions using pBGNA as template. PCR reactions were carried out using oligonucleotide primers designed from SEQ ID NO 2. The primers used were:

gnd fwd. primer: 5' ACT CTA GTC GGC CTA AAA TGG 3'
gnd rev. primer: 5' CAC ACA GGA AAC AGA TAT GAC 3'

PCR parameters were as follows:

35 cycles
95°C for 6 minutes
94°C for 1 minute
50°C for 1 minute
72°C for 45 seconds
1 mM $MgCl_2$
approx. 150-200ng pBGNA-DNA as template.

The PCR product obtained was cloned into the commercially available pGEM-T vector purchased from Promega Corp. (pGEM-T Easy Vector System 1, cat. no. A1360, Promega UK, Southampton) using E. coli strain JM109 (Yanisch-Perron et al. Gene, 33: 103-119 (1985)) as a host. The entire gnd gene was subsequently isolated from the pGEM T-vector on an EcoRI fragment and cloned into the lacZ EcoRI site of the E. coli - C. glutamicum shuttle vector pEC-T18mob2 (Figure 1), and designated pECgnd (Figure 2). Restriction enzyme analysis with AccI (Boehringer Mannheim GmbH, Germany) revealed the correct orientation (i. e. downstream the lac-Promotor) of the gnd gene in the lacZα gene of pEC-T18mob2.

Example 5

Preparation of amino acid producers with amplified 6-phosphogluconate dehydrogenase

Plasmid pECgnd from Example 3 was electroporated by the electroporation method of Tauch et al. (FEMS Microbiological Letters, 123:343-347 (1994)) in the strains Corynebacterium glutamicum DSM 5399 and DSM 5714. The strain DSM 5399 is a threonine producer described in EP-B-0358940. The strain DSM 5714 is a lysine producer described in EP-B-0435132. Selection of transformants was carried out by plating out the electroporation batch on LB agar (Sambrook et al., Molecular cloning: a laboratory manual. 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989), which had been supplemented with

25 mg/l kanamycin. The strains DSM5399/pECgnd and DSM5714/pECgnd were formed in this manner.

Example 6

Preparation of threonine

The C. glutamicum strain DSM5399/pECgnd obtained in Example 5 was cultured in a nutrient medium suitable for the production of threonine and the threonine content in the culture supernatant was determined.

For this, the strain was first incubated on an agar plate with the corresponding antibiotic (brain-heart agar with tetracycline (5 mg/l)) for 24 hours at 33°C. Starting from this agar plate culture, a preculture was seeded (10 ml medium in a 100 ml conical flask). Brain-heart broth (Merck, Darmstadt, Germany) was used as the medium for the preculture. Tetracycline (5 mg/l) was added to this medium. The preculture was incubated for 24 hours at 33°C at 240 rpm on a shaking machine. A main culture was seeded from this preculture such that the initial OD (660nm) of the main culture was 0.1. The medium MM-threonine was used for the main culture.

Medium MM-threonine:

CSL	5 g/l
MOPS	20 g/l
Glucose(autoclaved separately)	50g/l
Salts:	
$(NH_4)_2SO_4$	25 g/l
KH_2PO_4	0.1 g/l
$MgSO_4 * 7 H_2O$	1.0 g/l
$CaCl_2 * 2 H_2O$	10 mg/l
$FeSO_4 * 7 H_2O$	10 mg/l
$MnSO_4 * H_2O$	5.0mg/l
Biotin (sterile-filtered)	0.3 mg/l
Thiamine * HCl (sterile-filtered)	0.2 mg/l
$CaCO_3$	25 g/l

The CSL (corn steep liquor), MOPS (morpholinopropanesulfonic acid) and the salt solution were brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions were then added, as well as the $CaCO_3$ autoclaved in the dry state.

Culturing is carried out in a 10 ml volume in a 100 ml conical flask with baffles. Tetracycline (5 mg/l) was added. Culturing was carried out at 33°C and 80% atmospheric humidity.

After 48 hours, the OD was determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The concentration of threonine formed was determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivatization with ninhydrin detection.

The result of the experiment is shown in Table 1.

Table 1

Strain	OD(660)	L-Threonin g/l
DSM5399/pECgnd	11.9	1.29
DSM5399	11.8	0.33

Example 7

Preparation of lysine

The C. glutamicum strain DSM5714/pECgnd obtained in Example 5 was cultured in a nutrient medium suitable for the production of lysine and the lysine content in the culture supernatant was determined.

For this, the strain was first incubated on an agar plate with the corresponding antibiotic (brain-heart agar with tetracycline (5 mg/l)) for 24 hours at 33°C. Starting from this agar plate culture, a preculture was seeded (10 ml medium in a 100 ml conical flask). The complete medium CgIII was used as the medium for the preculture.

Medium Cg III

NaCl	2.5 g/l
Bacto-Peptone	10 g/l
Bacto-Yeast extract	10 g/l
Glucose (autoclaved separately)	2% (w/v)

The pH was brought to pH 7.4

Tetracycline (5 mg/l) was added to this medium. The preculture was incubated for 24 hours at 33°C at 240 rpm on a shaking machine. A main culture was seeded from this preculture such that the initial OD (660nm) of the main culture was 0.05. Medium MM was used for the main culture.

Medium MM	
CSL (corn steep liquor)	5 g/l
MOPS (morpholinopropanesulfonic acid)	20 g/l
Glucose (autoclaved separately)	50g/l
$(NH_4)_2SO_4$	
KH_2PO_4	25 g/l
$MgSO_4 * 7 H_2O$	0.1 g/l
$CaCl_2 * 2 H_2O$	1.0 g/l
$FeSO_4 * 7 H_2O$	10 mg/l
$MnSO_4 * H_2O$	10 mg/l
Biotin (sterile-filtered)	0.3 mg/l
Thiamine * HCl (sterile-filtered)	0.2 mg/l
L-Leucine (sterile-filtered)	0.1 g/l
$CaCO_3$	25 g/l

The CSL, MOPS and the salt solution were brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions were then added, as well as the $CaCO_3$ autoclaved in the dry state.

Culturing is carried out in a 10 ml volume in a 100 ml conical flask with baffles. Tetracycline (5 mg/l) was added. Culturing was carried out at 33°C and 80% atmospheric humidity.

After 48 hours, the OD was determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, München). The amount of lysine formed was determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivatization with ninhydrin detection.

The result of the experiment is shown in Table 2.

Table 2

Strain	OD(660)	Lysine HCl g/l
DSM5715/pECgnd	7.7	14.7
DSM5715	7.1	13.7

Example 8

Preparation of a genomic cosmid gene library from Corynebacterium glutamicum ATCC 13032

Chromosomal DNA from Corynebacterium glutamicum ATCC 13032 was isolated as described by Tauch et al., (1995, Plasmid 33:168-179), and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Code no. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, Product Description SAP, Code no. 1758250). The DNA of the cosmid vector SuperCos1 (Wahl et al. (1987) Proceedings of the National Academy of Sciences USA 84:2160-2164), obtained from Stratagene (La Jolla, USA, Product Description SuperCos1 Cosmid Vektor Kit, Code no. 251301) was cleaved with the restriction enzyme XbaI (Amersham Pharmacia, Freiburg, Germany, Product Description XbaI, Code no. 27-

0948-02) and likewise dephosphorylated with shrimp alkaline phosphatase. The cosmid DNA was then cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Code no. 27-0868-04). The cosmid DNA treated in this manner was mixed with the treated ATCC13032 DNA and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04). The ligation mixture was then packed in phages with the aid of Gigapack II XL Packing Extracts (Stratagene, La Jolla, USA, Product Description Gigapack II XL Packing Extract, Code no. 200217). For infection of the E. coli strain NM554 (Raleigh et al. 1988, Nucleic Acid Research 16:1563-1575) the cells were taken up in 10 mM $MgSO_4$ and mixed with an aliquot of the phage suspension. The infection and titering of the cosmid library were carried out as described by Sambrook et al. (1989, Molecular Cloning: A laboratory Manual, Cold Spring Harbor), the cells being plated out on LB agar (Lennox, 1955, Virology 1:190) + 100 µg/ml ampicillin. After incubation overnight at 37°C, recombinant individual clones were selected.

Example 9

Isolation and sequencing of the poxB gene

The cosmid DNA of an individual colony (Example 8) was isolated with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Product No. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, Product Description SAP, Product No. 1758250). After separation by gel electrophoresis, the cosmid fragments in the size range of 1500 to 2000 bp were isolated with the QiaExII Gel Extraction Kit (Product No.

20021, Qiagen, Hilden, Germany). The DNA of the sequencing vector pZero-1, obtained from Invitrogen (Groningen, Holland, Product Description Zero Background Cloning Kit, Product No. K2500-01) was cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Product No. 27-0868-04). The ligation of the cosmid fragments in the sequencing vector pZero-1 was carried out as described by Sambrook et al. (1989, Molecular Cloning: A laboratory Manual, Cold Spring Harbor), the DNA mixture being incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). This ligation mixture was then electroporated (Tauch et al. 1994, FEMS Microbiol Letters, 123:343-7) into the E. coli strain DH5αMCR (Grant, 1990, Proceedings of the National Academy of Sciences U.S.A., 87:4645-4649) and plated out on LB agar (Lennox, 1955, Virology, 1:190) with 50 µg/ml zeocin. The plasmid preparation of the recombinant clones was carried out with Biorobot 9600 (Product No. 900200, Qiagen, Hilden, Germany). The sequencing was carried out by the dideoxy chain-stopping method of Sanger et al. (1977, Proceedings of the National Academies of Sciences U.S.A., 74:5463-5467) with modifications according to Zimmermann et al. (1990, Nucleic Acids Research, 18:1067). The "RR dRhodamin Terminator Cycle Sequencing Kit" from PE Applied Biosystems(Product No. 403044, Weiterstadt, Germany) was used. The separation by gel electrophoresis and analysis of the sequencing reaction were carried out in a "Rotiphoresis NF Acrylamide/Bisacrylamide" Gel (29:1) (Product No. A124.1, Roth, Karlsruhe, Germany) with the "ABI Prism 377" sequencer from PE Applied Biosystems (Weiterstadt, Germany).

The raw sequence data obtained were then processed using the Staden program package (1986, Nucleic Acids Research, 14:217-231) version 97-0. The individual sequences of the pZero1 derivatives were assembled to a continuous contig. The computer-assisted coding region analysis were prepared

with the XNIP program (Staden, 1986, Nucleic Acids Research 14:217-231). Further analyses were carried out with the "BLAST search program" (Altschul et al., 1997, Nucleic Acids Research 25:3389-3402), against the non-redundant databank of the "National Center for Biotechnology Information" (NCBI, Bethesda, MD, USA).

The resulting nucleotide sequence is shown in SEQ ID No. 4. Analysis of the nucleotide sequence showed an open reading frame of 1737 base pairs, which was called the poxB gene. The poxB gene codes for a polypeptide of 579 amino acids (SEQ ID NO. 5).

Example 10

Preparation of an integration vector for integration mutagenesis of the poxB gene

From the strain ATCC 13032, chromosomal DNA was isolated by the method of Eikmanns et al. (Microbiology 140: 1817 - 1828 (1994)). On the basis of the sequence of the poxB gene known for C. glutamicum from Example 9, the following oligonucleotides were chosen for the polymerase chain reaction:

poxBint1:
5` TGC GAG ATG GTG AAT GGT GG 3`
poxBint2:
5` GCA TGA GGC AAC GCA TTA GC 3`

The primers shown were synthesized by MWG Biotech (Ebersberg, Germany) and the PCR reaction was carried out by the standard PCR method of Innis et al. (PCR protocols. A guide to methods and applications, 1990, Academic Press) with Pwo-Polymerase from Boehringer. With the aid of the polymerase chain reaction, a DNA fragment approx. 0.9 kb in size was isolated, this carrying an internal fragment of the poxB gene and being shown in SEQ ID No. 6.

The amplified DNA fragment was ligated with the TOPO TA Cloning Kit from Invitrogen Corporation (Carlsbad, CA, USA; Catalogue Number K4500-01) in the vector pCR2.1-TOPO (Mead at al. (1991) Bio/Technology 9:657-663). The E. coli Stamm DH5α was then electroporated with the ligation batch (Hanahan, In: DNA cloning. A practical approach. Vol.I. IRL-Press, Oxford, Washington DC, USA, 1985). Selection for plasmid-carrying cells was made by plating out the transformation batch on LB agar (Sambrook et al., Molecular cloning: a laboratory manual. 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989), which had been supplemented with 25 mg/l kanamycin. Plasmid DNA was isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction with the restriction enzyme EcoRI and subsequent agarose gel electrophoresis (0.8%). The plasmid was called pCR2.1poxBint (Figure 4).

Plasmid pCR2.1poxBint has been deposited in the form of the strain Escherichia coli DH5α/pCR2.1poxBint as DSM 13114 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty.

Example 11

Integration mutagenesis of the poxB gene in the lysine producer DSM 5715

The vector pCR2.1poxBint mentioned in Example 10 was electroporated by the electroporation method of Tauch et al.(FEMS Microbiological Letters, 123:343-347 (1994)) in Corynebacterium glutamicum DSM 5715. Strain DSM 5715 is an AEC-resistant lysine producer. The vector pCR2.1poxBint cannot replicate independently in DSM5715 and is retained in the cell only if it has integrated into the chromosome of DSM 5715. Selection of clones with pCR2.1poxBint

integrated into the chromosome was carried out by plating out the electroporation batch on LB agar (Sambrook et al., Molecular cloning: a laboratory manual. 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.), which had been supplemented with 15 mg/l kanamycin. For detection of the integration, the poxBint fragment was labelled with the Dig hybridization kit from Boehringer by the method of "The DIG System Users Guide for Filter Hybridization" of Boehringer Mannheim GmbH (Mannheim, Germany, 1993). Chromosomal DNA of a potential integrant was isolated by the method of Eikmanns et al. (Microbiology 140: 1817 - 1828 (1994)) and in each case cleaved with the restriction enzymes SalI, SacI and HindIII. The fragments formed were separated by agarose gel electrophoresis and hybridized at 68°C with the Dig hybrization kit from Boehringer. The plasmid pCR2.1poxBint mentioned in Example 9 had been inserted into the chromosome of DSM5715 within the chromosomal poxB gene. The strain was called DSM5715::pCR2.1poxBint.

Example 12

Effect of over-expression of the gnd gene with simultaneous elimination of the poxB gene on the preparation of lysine

12.1 Preparation of the strain DSM5715::pCR2.1poxBint/pECgnd

The strain DSM5715::pCR2.1poxBint was transformed with the plasmid pECgnd using the electroporation method described by Liebl et al., (FEMS Microbiology Letters, 53:299-303 (1989)). Selection of the transformants took place on LBHIS agar comprising 18.5 g/l brain-heart infusion broth, 0.5 M sorbitol, 5 g/l Bacto-tryptone, 2.5 g/l Bacto-yeast extract, 5 g/l NaCl and 18 g/l Bacto-agar, which had been supplemented with 5 mg/l tetracycline and 25 mg/l kanamycin. Incubation was carried out for 2 days at 33°C.

Plasmid DNA was isolated in each case from a transformant by conventional methods (Peters-Wendisch et al., 1998, Microbiology 144, 915 -927), cleaved with the restriction endonuclease AccI, and the plasmid was checked by subsequent agarose gel electrophoresis. The strain obtained in this way was called DSM5715:pCR2.1poxBint/pECgnd.

12.2 Preparation of L-lysine

The C. glutamicum strain DSM5715::pCR2.1poxBint/pECgnd obtained in Example 12.1 was cultured in a nutrient medium suitable for the production of lysine and the lysine content in the culture supernatant was determined.

For this, the strain was first incubated on an agar plate with the corresponding antibiotic (brain-heart agar with tetracycline (5 mg/l) and kanamycin (25 mg/l)) for 24 hours at 33°C. The cultures of the comparison strains were supplemented according to their resistance to antibiotics. Starting from this agar plate culture, a preculture was seeded (10 ml medium in a 100 ml conical flask). The complete medium CgIII was used as the medium for the preculture.

Medium Cg III

NaCl	2.5 g/l
Bacto-Peptone	10 g/l
Bacto-Yeast extract	10 g/l
Glucose (autoclaved separately)	2% (w/v)

The pH was brought to pH 7.4

Tetracycline (5 mg/l) and kanamycin (25 mg/l) were added to this. The preculture was incubated for 16 hours at 33°C at 240 rpm on a shaking machine. A main culture was seeded

from this preculture such that the initial OD (660nm) of the main culture was 0.1. Medium MM was used for the main culture.

Medium MM

CSL (corn steep liquor)	5 g/l
MOPS (morpholinopropanesulfonic acid)	20 g/l
Glucose (autoclaved separately)	58 g/l
$(NH_4)_2SO_4$	25 g/l
KH_2PO_4	0.1 g/l
$MgSO_4 * 7 H_2O$	1.0 g/l
$CaCl_2 * 2 H_2O$	10 mg/l
$FeSO_4 * 7 H_2O$	10 mg/l
$MnSO_4 * H_2O$	5.0mg/l
Biotin (sterile-filtered)	0.3 mg/l
Thiamine * HCl (sterile-filtered)	0.2 mg/l
L-Leucine (sterile-filtered)	0.1 g/l
$CaCO_3$	25 g/l

The CSL, MOPS and the salt solution were brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions were then added, as well as the $CaCO_3$ autoclaved in the dry state.

Culturing is carried out in a 10 ml volume in a 100 ml conical flask with baffles. Tetracycline (5 mg/l) and kanamycin (25 mg/l) were added. Culturing was carried out at 33°C and 80% atmospheric humidity.

After 72 hours, the OD was determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, München). The amount of lysine formed was determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivatization with ninhydrin detection.

The result of the experiment is shown in Table 3.

Table 3

Strain	OD	L-Lysine HCl g/l
DSM5715	10.8	16.0
DSM5715/pECgnd	7.6	16.5
DSM5715::pCR2.1poxBint	7.1	16.7
DSM5715::pCR2.1poxBint/pECgnd	7.2	17.1

Patent claims

1. A process for the preparation of L-amino acids by fermentation of coryneform bacteria
which comprises
carrying out the following steps:
a) fermentation of the desired L-amino acid-producing bacteria in which at least the gnd gene is amplified,
b) concentration of the L-amino acid in the medium or in the cells of the bacteria and
c) isolation of the L-amino acid produced.

2. The process as claimed in claim 1,
wherein
bacteria in which further genes of the biosynthesis pathway of the desired L-amino acid are additionally amplified, in particular over-expressed, are employed.

3. The process as claimed in claim 1,
wherein
coryneform bacteria which prepare L-threonine, L-lysine, L-isoleucine or L-tryptophan are used.

4. The process as claimed in claim 3,
wherein
coryneform bacteria which prepare L-lysine are used.

5. A process for the fermentative preparation of L-lysine as claimed in claim 2,
wherein
in the coryneform microorganisms which in particular already produce L-lysine, one or more genes chosen from the group consisting of

5.1 the dapA gene which codes for dihydrodipicolinate synthase,

5.2 the lysC gene which codes for a feed back resistant aspartate kinase,

5.3 the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase,

5.4 the pyc gene which codes for pyruvate carboxylase,

5.5 the tkt gene which codes for transketolase,

5.6 the zwf gene which codes for glucose 6-phosphate dehydrogenase,

5.7 the lysE gene which codes for lysine export,

5.8 the zwa1 gene

5.9 the eno gene which codes for enolase

is or are amplified, in particular over-expressed, at the same time.

6. A process for the fermentative preparation of L-threonine as claimed in claim 2,
w h e r e i n
in the coryneform microorganisms which in particular already produce L-threonine, one or more genes chosen from the group consisting of

6.1 the hom gene which codes for homoserine dehydrogenase or the hom^{dr} allele which codes for a "feed back resistant" homoserine dehydrogenase,

6.2 the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase,

6.3 the pyc gene which codes for pyruvate carboxylase,

6.4 the mqo gene which codes for malate:quinone oxidoreductase,

6.5 the tkt gene which codes for transketolase,

6.6 the zwf gene which codes for glucose 6-phosphate dehydrogenase,

6.7 the thrE gene which codes for threonine export,

6.8 the zwa1 gene,

6.9 the eno gene which codes for enolase

is or are amplified, in particular over-expressed, at the same time.

7. The process as claimed in claim 2,
w h e r e i n
for the preparation of L-amino acids, in particular L-lysine or L-threonine, bacteria in which one or more genes chosen from the group consisting of,

7.1 the pck gene which codes for phosphoenol pyruvate carboxykinase

7.2 the pgi gene which codes for glucose 6-phosphate isomerase

7.3 the poxB gene which codes for pyruvate oxidase or

7.4 the zwa2 gene

is or are attenuated at the same time, are fermented.

8. The process as claimed in claims 2 to 6,
w h e r e i n
to achieve the amplification, the number of copies of the genes or nucleotide sequences is increased by transformation of the microorganisms with plasmid

vectors which carry these genes or nucleotide sequences.

9. The plasmid vector pEC-T18mob2 deposited under the designation DSM 13244 in E. coli K-12 DH5α, shown in Figure 1.

10. A coryneform microorganism, in particular of the genus Corynebacterium, transformed by the introduction of the plasmid vector as claimed in claim 9, which additionally contains the gnd gene.

Figure 1: Map of the plasmid pEC-T18mob2

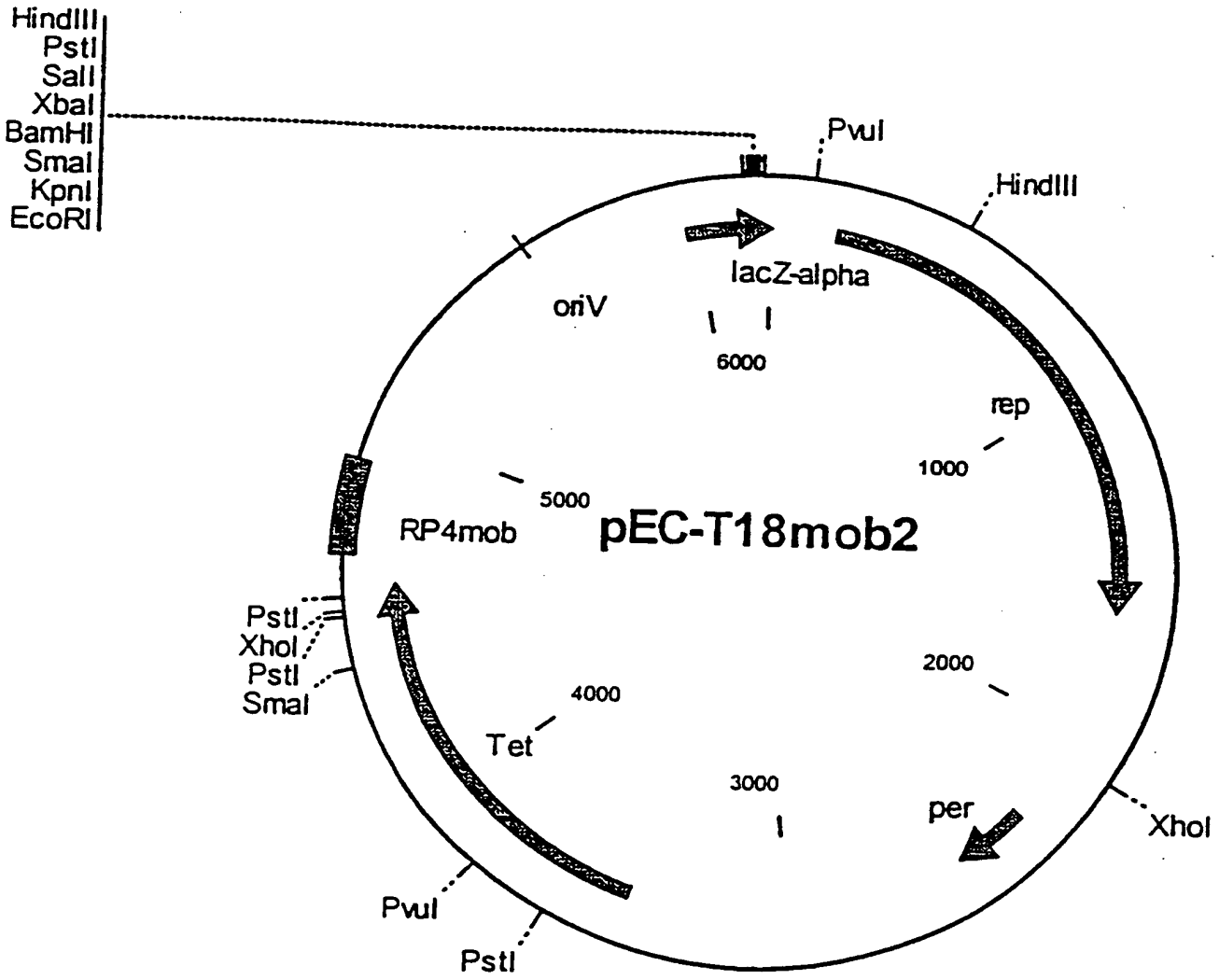


Figure 2: Map of the plasmid pECgnd

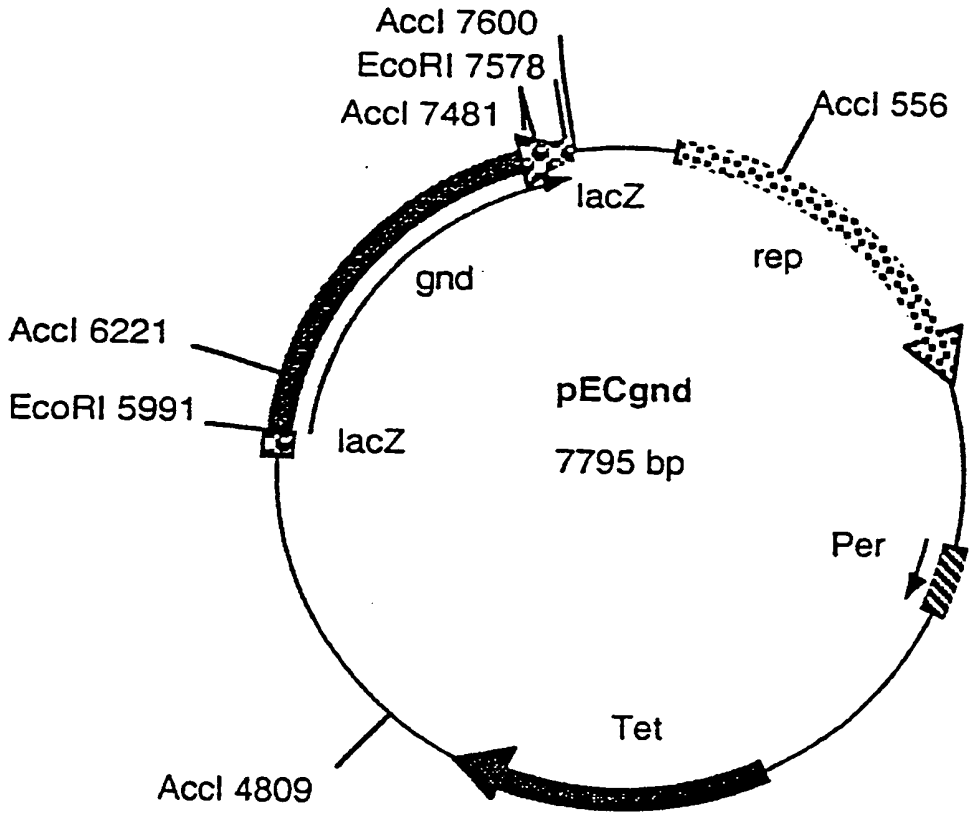


Figure 3: Map of the plasmid pBGNA

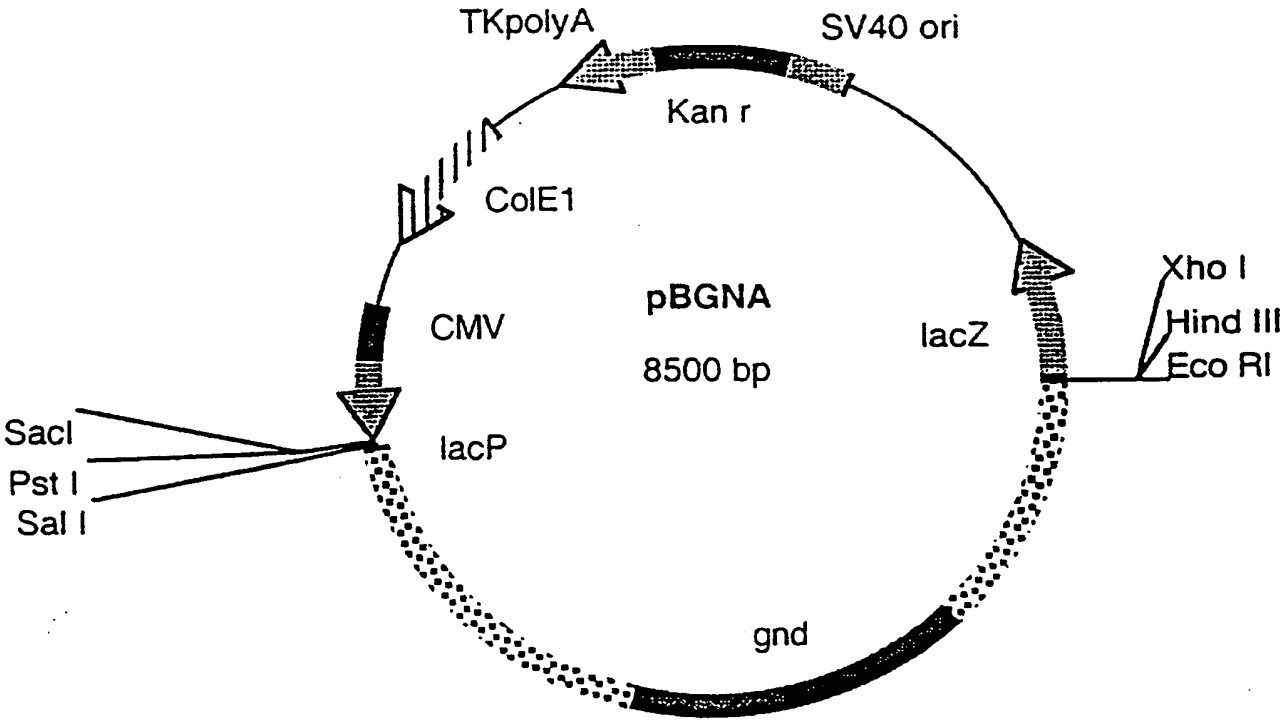
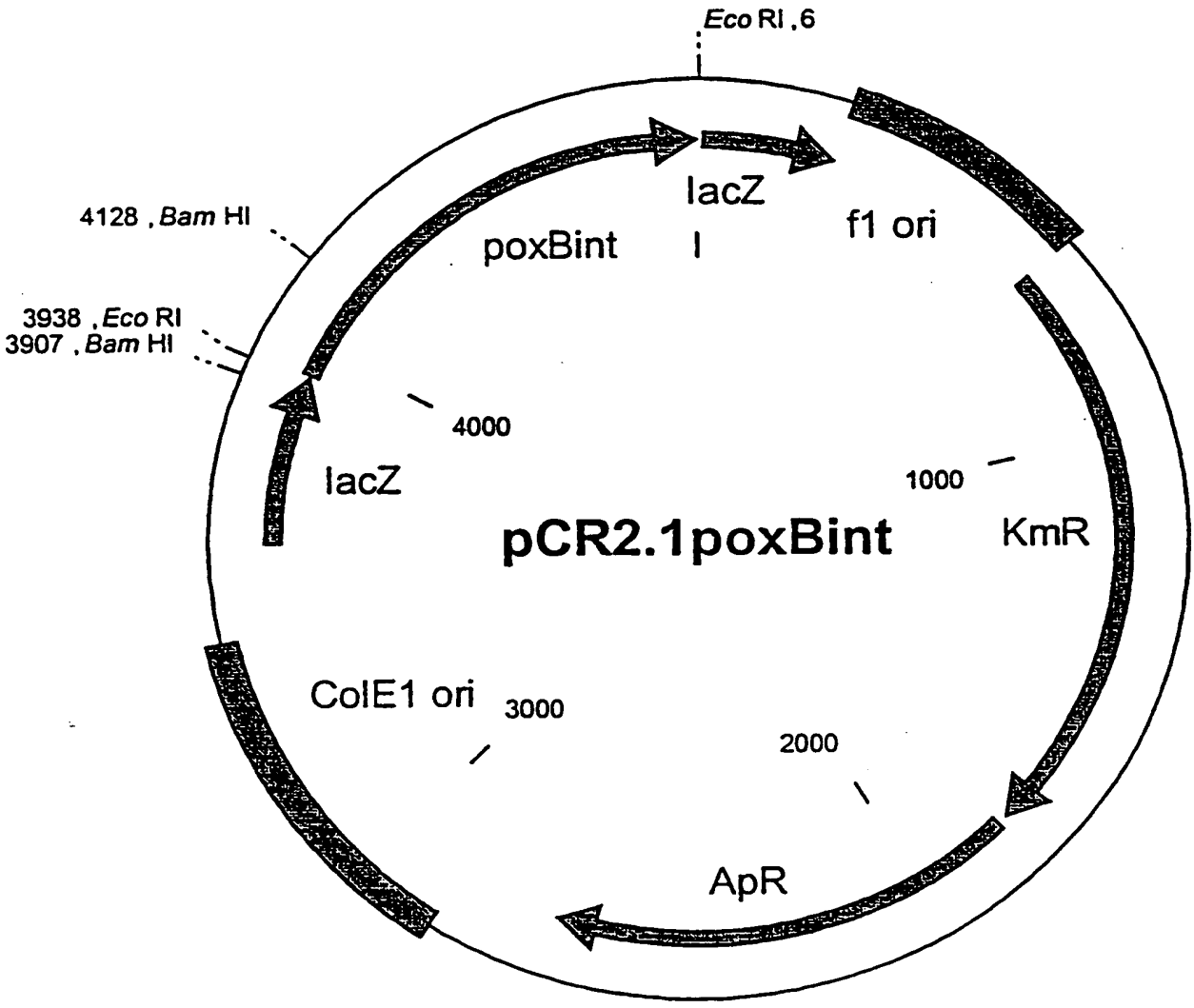


Figure 4: Map of the plasmid pCR2.1poxBint



SEQUENCE PROTOCOL

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Degussa-Hüls AG

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     35                  40                  45

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 50                  55                  60                  65

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Pro Ser Ala Thr Val Glu Glu Phe Val Ala Ser Leu Glu Lys Pro Arg
                 70                  75                  80

cgc gcc atc atc atg gtt cag gct ggt aac gcc acc gac gca gtc atc   764
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    115                 120                 125

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Ala Arg Gly Leu His Phe Val Gly Ala Gly Ile Ser Gly Gly Glu Glu
130                 135                 140                 145

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Gly Ala Leu Asn Gly Pro Ser Ile Met Pro Gly Gly Pro Ala Lys Ser
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Tyr Glu Ser Leu Gly Pro Leu Leu Glu Ser Ile Ala Ala Asn Val Asp
            165                 170                 175

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210                 215                 220                 225

gaa atc gct gag gtt ttc aag gaa tgg aac gca ggc gac ctg gat tcc   1196
Glu Ile Ala Glu Val Phe Lys Glu Trp Asn Ala Gly Asp Leu Asp Ser
                230                 235                 240

tac ctc atc gaa atc acc gca gag gtt ctc tcc cag gtg gat gct gaa   1244
Tyr Leu Ile Glu Ile Thr Ala Glu Val Leu Ser Gln Val Asp Ala Glu
            245                 250                 255

acc ggc aag cca cta atc gac gtc atc gtt gac gct gca ggt cag aag   1292
Thr Gly Lys Pro Leu Ile Asp Val Ile Val Asp Ala Ala Gly Gln Lys
        260                 265                 270
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ggc acc ggc aag tgg act gtc aag gct gct ctt gat ctg ggt att gct   1340
Gly Thr Gly Lys Trp Thr Val Lys Ala Ala Leu Asp Leu Gly Ile Ala
    275                 280                 285

acc acc ggc atc ggc gaa cgt gtt ttc gca cgt gca ctc tcc ggc gca   1388
Thr Thr Gly Ile Gly Glu Arg Val Phe Ala Arg Ala Leu Ser Gly Ala
290                 295                 300                 305

acc agc cag cgc gct gca gca cag ggc aac cta cct gca ggt gtc ctc   1436
Thr Ser Gln Arg Ala Ala Ala Gln Gly Asn Leu Pro Ala Gly Val Leu
                310                 315                 320

acc gat ctg gaa gca ctt ggc gtg gac aag gca cag ttc gtc gaa gga   1484
Thr Asp Leu Glu Ala Leu Gly Val Asp Lys Ala Gln Phe Val Glu Gly
            325                 330                 335

ctt cgc cgt gca ctg tac gca tcc aag ctt gtt gct tac gca cag ggc   1532
Leu Arg Arg Ala Leu Tyr Ala Ser Lys Leu Val Ala Tyr Ala Gln Gly
        340                 345                 350

ttc gac gag atc aag gct ggc tcc gac gag aac aac tgg gac gtt gac   1580
Phe Asp Glu Ile Lys Ala Gly Ser Asp Glu Asn Asn Trp Asp Val Asp
    355                 360                 365

cct cgc gac ctc gct acc atc tgg cgc ggc ggc tgc atc att cgc gct   1628
Pro Arg Asp Leu Ala Thr Ile Trp Arg Gly Gly Cys Ile Ile Arg Ala
370                 375                 380                 385

aag ttc ctc aac cgc atc gtc gaa gca tac gat gca aac gct gaa ctt   1676
Lys Phe Leu Asn Arg Ile Val Glu Ala Tyr Asp Ala Asn Ala Glu Leu
                390                 395                 400

gag tcc ctg ctg ctc gat cct tac ttc aag agc gag ctc ggc gac ctc   1724
Glu Ser Leu Leu Leu Asp Pro Tyr Phe Lys Ser Glu Leu Gly Asp Leu
            405                 410                 415

atc gat tca tgg cgt cgc gtg att gtc acc gcc acc cag ctt ggc ctg   1772
Ile Asp Ser Trp Arg Arg Val Ile Val Thr Ala Thr Gln Leu Gly Leu
        420                 425                 430

cca atc cca gtg ttc gct tcc tcc ctg tcc tac tac gac agc ctg cgt   1820
Pro Ile Pro Val Phe Ala Ser Ser Leu Ser Tyr Tyr Asp Ser Leu Arg
    435                 440                 445

gca gag cgt ctg cca gca gcc ctg atc cac tagtgtcgac ctgcaggcgc     1870
Ala Glu Arg Leu Pro Ala Ala Leu Ile His
450                 455

gcgagctcca gcttttgttc cctttagtga gggttaattt cgagcttggc gtaatcaagg 1930

tcatagctgt ttcctgtgtg aaattgttat ccgctcacaa ttccacacaa tatacgagcc 1990

ggaagtataa agtgtaaagc ctggggtgcc taatgagtga gctaactcac agtaattgcg 2050

gctagcggat ctgacggttc actaaaccag ctctgcttat atagacctcc caccgtacac 2110

gcctaccgcc catttgcgtc aatggggcgg agttgttacg acattttgga aagtcccgtt 2170

gattttggtg ccaaaacaaa ctcccattga cgtcaatggg gtggagactt ggaaatcccc 2230

gtgagtcaaa ccgctatcca cgcccattga tgtactgcca aaaccgcatc accatggtaa 2290
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tagcgatgac taatacgtag atgtactgcc aagtaggaaa gtccc 2335

<210> 3
<211> 459
<212> PRT
<213> Corynebacterium glutamicum

<400> 3

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Met Pro Ser Ser Thr Ile Asn Asn Met Thr Asn Gly Asp Asn Leu Ala
 1               5                  10                  15

Gln Ile Gly Val Val Gly Leu Ala Val Met Gly Ser Asn Leu Ala Arg
            20                  25                  30

Asn Phe Ala Arg Asn Gly Asn Thr Val Ala Val Tyr Asn Arg Ser Thr
        35                  40                  45

Asp Lys Thr Asp Lys Leu Ile Ala Asp His Gly Ser Glu Gly Asn Phe
    50                  55                  60

Ile Pro Ser Ala Thr Val Glu Glu Phe Val Ala Ser Leu Glu Lys Pro
65                  70                  75                  80

Arg Arg Ala Ile Ile Met Val Gln Ala Gly Asn Ala Thr Asp Ala Val
                85                  90                  95

Ile Asn Gln Leu Ala Asp Ala Met Asp Glu Gly Asp Ile Ile Ile Asp
            100                 105                 110

Gly Gly Asn Ala Leu Tyr Thr Asp Thr Ile Arg Arg Glu Lys Glu Ile
        115                 120                 125

Ser Ala Arg Gly Leu His Phe Val Gly Ala Gly Ile Ser Gly Gly Glu
    130                 135                 140

Glu Gly Ala Leu Asn Gly Pro Ser Ile Met Pro Gly Gly Pro Ala Lys
145                 150                 155                 160

Ser Tyr Glu Ser Leu Gly Pro Leu Leu Glu Ser Ile Ala Ala Asn Val
                165                 170                 175

Asp Gly Thr Pro Cys Val Thr His Ile Gly Pro Asp Gly Ala Gly His
            180                 185                 190

Phe Val Lys Met Val His Asn Gly Ile Glu Tyr Ala Asp Met Gln Val
        195                 200                 205

Ile Gly Glu Ala Tyr His Leu Leu Pro Tyr Ala Ala Gly Met Gln Pro
    210                 215                 220

Ala Glu Ile Ala Glu Val Phe Lys Glu Trp Asn Ala Gly Asp Leu Asp
225                 230                 235                 240

Ser Tyr Leu Ile Glu Ile Thr Ala Glu Val Leu Ser Gln Val Asp Ala
                245                 250                 255

Glu Thr Gly Lys Pro Leu Ile Asp Val Ile Val Asp Ala Ala Gly Gln
            260                 265                 270

Lys Gly Thr Gly Lys Trp Thr Val Lys Ala Ala Leu Asp Leu Gly Ile
        275                 280                 285
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Ala Thr Thr Gly Ile Gly Glu Arg Val Phe Ala Arg Ala Leu Ser Gly
290 295 300

Ala Thr Ser Gln Arg Ala Ala Ala Gln Gly Asn Leu Pro Ala Gly Val
305 310 315 320

Leu Thr Asp Leu Glu Ala Leu Gly Val Asp Lys Ala Gln Phe Val Glu
325 330 335

Gly Leu Arg Arg Ala Leu Tyr Ala Ser Lys Leu Val Ala Tyr Ala Gln
340 345 350

Gly Phe Asp Glu Ile Lys Ala Gly Ser Asp Glu Asn Asn Trp Asp Val
355 360 365

Asp Pro Arg Asp Leu Ala Thr Ile Trp Arg Gly Gly Cys Ile Ile Arg
370 375 380

Ala Lys Phe Leu Asn Arg Ile Val Glu Ala Tyr Asp Ala Asn Ala Glu
385 390 395 400

Leu Glu Ser Leu Leu Leu Asp Pro Tyr Phe Lys Ser Glu Leu Gly Asp
405 410 415

Leu Ile Asp Ser Trp Arg Arg Val Ile Val Thr Ala Thr Gln Leu Gly
420 425 430

Leu Pro Ile Pro Val Phe Ala Ser Ser Leu Ser Tyr Tyr Asp Ser Leu
435 440 445

Arg Ala Glu Arg Leu Pro Ala Ala Leu Ile His
450 455

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<211> 2160
<212> DNA
<213> Corynebacterium glutamicum

<220>
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<222> (327)..(2063)
<223> poxB

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cgaggcgacc agacaggcgt gcccacgatg tttaaatagg cgatcggtgg gcatctgtgt 120

ttggtttcga cgggctgaaa ccaaaccaga ctgcccagca acgacggaaa tcccaaaagt 180

gggcatccct gtttggtacc gagtacccac ccgggcctga aactccctgg caggcgggcg 240

aagcgtggca acaactggaa tttaagagca caattgaagt cgcaccaagt taggcaacac 300

aatagccata acgttgagga gttcag atg gca cac agc tac gca gaa caa tta 353
Met Ala His Ser Tyr Ala Glu Gln Leu
1 5

att gac act ttg gaa gct caa ggt gtg aag cga att tat ggt ttg gtg 401
Ile Asp Thr Leu Glu Ala Gln Gly Val Lys Arg Ile Tyr Gly Leu Val
10 15 20 25

```
ggt gac agc ctt aat ccg atc gtg gat gct gtc cgc caa tca gat att   449
Gly Asp Ser Leu Asn Pro Ile Val Asp Ala Val Arg Gln Ser Asp Ile
                30                  35                  40

gag tgg gtg cac gtt cga aat gag gaa gcg gcg gcg ttt gca gcc ggt   497
Glu Trp Val His Val Arg Asn Glu Glu Ala Ala Ala Phe Ala Ala Gly
            45                  50                  55

gcg gaa tcg ttg atc act ggg gag ctg gca gta tgt gct gct tct tgt   545
Ala Glu Ser Leu Ile Thr Gly Glu Leu Ala Val Cys Ala Ala Ser Cys
        60                  65                  70

ggt cct gga aac aca cac ctg att cag ggt ctt tat gat tcg cat cga   593
Gly Pro Gly Asn Thr His Leu Ile Gln Gly Leu Tyr Asp Ser His Arg
    75                  80                  85

aat ggt gcg aag gtg ttg gcc atc gct agc cat att ccg agt gcc cag   641
Asn Gly Ala Lys Val Leu Ala Ile Ala Ser His Ile Pro Ser Ala Gln
90                  95                  100                 105

att ggt tcg acg ttc ttc cag gaa acg cat ccg gag att ttg ttt aag   689
Ile Gly Ser Thr Phe Phe Gln Glu Thr His Pro Glu Ile Leu Phe Lys
                110                 115                 120

gaa tgc tct ggt tac tgc gag atg gtg aat ggt ggt gag cag ggt gaa   737
Glu Cys Ser Gly Tyr Cys Glu Met Val Asn Gly Gly Glu Gln Gly Glu
            125                 130                 135

cgc att ttg cat cac gcg att cag tcc acc atg gcg ggt aaa ggt gtg   785
Arg Ile Leu His His Ala Ile Gln Ser Thr Met Ala Gly Lys Gly Val
        140                 145                 150

tcg gtg gta gtg att cct ggt gat atc gct aag gaa gac gca ggt gac   833
Ser Val Val Val Ile Pro Gly Asp Ile Ala Lys Glu Asp Ala Gly Asp
    155                 160                 165

ggt act tat tcc aat tcc act att tct tct ggc act cct gtg gtg ttc   881
Gly Thr Tyr Ser Asn Ser Thr Ile Ser Ser Gly Thr Pro Val Val Phe
170                 175                 180                 185

ccg gat cct act gag gct gca gcg ctg gtg gag gcg att aac aac gct   929
Pro Asp Pro Thr Glu Ala Ala Ala Leu Val Glu Ala Ile Asn Asn Ala
                190                 195                 200

aag tct gtc act ttg ttc tgc ggt gcg ggc gtg aag aat gct cgc gcg   977
Lys Ser Val Thr Leu Phe Cys Gly Ala Gly Val Lys Asn Ala Arg Ala
            205                 210                 215

cag gtg ttg gag ttg gcg gag aag att aaa tca ccg atc ggg cat gcg   1025
Gln Val Leu Glu Leu Ala Glu Lys Ile Lys Ser Pro Ile Gly His Ala
        220                 225                 230

ctg ggt ggt aag cag tac atc cag cat gag aat ccg ttt gag gtc ggc   1073
Leu Gly Gly Lys Gln Tyr Ile Gln His Glu Asn Pro Phe Glu Val Gly
    235                 240                 245

atg tct ggc ctg ctt ggt tac ggc gcc tgc gtg gat gcg tcc aat gag   1121
Met Ser Gly Leu Leu Gly Tyr Gly Ala Cys Val Asp Ala Ser Asn Glu
250                 255                 260                 265

gcg gat ctg ctg att cta ttg ggt acg gat ttc cct tat tct gat ttc   1169
Ala Asp Leu Leu Ile Leu Leu Gly Thr Asp Phe Pro Tyr Ser Asp Phe
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                    270                 275                 280

ctt cct aaa gac aac gtt gcc cag gtg gat atc aac ggt gcg cac att   1217
Leu Pro Lys Asp Asn Val Ala Gln Val Asp Ile Asn Gly Ala His Ile
            285                 290                 295

ggt cga cgt acc acg gtg aag tat ccg gtg acc ggt gat gtt gct gca   1265
Gly Arg Arg Thr Thr Val Lys Tyr Pro Val Thr Gly Asp Val Ala Ala
        300                 305                 310

aca atc gaa aat att ttg cct cat gtg aag gaa aaa aca gat cgt tcc   1313
Thr Ile Glu Asn Ile Leu Pro His Val Lys Glu Lys Thr Asp Arg Ser
    315                 320                 325

ttc ctt gat cgg atg ctc aag gca cac gag cgt aag ttg agc tcg gtg   1361
Phe Leu Asp Arg Met Leu Lys Ala His Glu Arg Lys Leu Ser Ser Val
330                 335                 340                 345

gta gag acg tac aca cat aac gtc gag aag cat gtg cct att cac cct   1409
Val Glu Thr Tyr Thr His Asn Val Glu Lys His Val Pro Ile His Pro
                350                 355                 360

gaa tac gtt gcc tct att ttg aac gag ctg gcg gat aag gat gcg gtg   1457
Glu Tyr Val Ala Ser Ile Leu Asn Glu Leu Ala Asp Lys Asp Ala Val
            365                 370                 375

ttt act gtg gat acc ggc atg tgc aat gtg tgg cat gcg agg tac atc   1505
Phe Thr Val Asp Thr Gly Met Cys Asn Val Trp His Ala Arg Tyr Ile
        380                 385                 390

gag aat ccg gag gga acg cgc gac ttt gtg ggt tca ttc cgc cac ggc   1553
Glu Asn Pro Glu Gly Thr Arg Asp Phe Val Gly Ser Phe Arg His Gly
    395                 400                 405

acg atg gct aat gcg ttg cct cat gcg att ggt gcg caa agt gtt gat   1601
Thr Met Ala Asn Ala Leu Pro His Ala Ile Gly Ala Gln Ser Val Asp
410                 415                 420                 425

cga aac cgc cag gtg atc gcg atg tgt ggc gat ggt ggt ttg ggc atg   1649
Arg Asn Arg Gln Val Ile Ala Met Cys Gly Asp Gly Gly Leu Gly Met
                430                 435                 440

ctg ctg ggt gag ctt ctg acc gtt aag ctg cac caa ctt ccg ctg aag   1697
Leu Leu Gly Glu Leu Leu Thr Val Lys Leu His Gln Leu Pro Leu Lys
            445                 450                 455

gct gtg gtg ttt aac aac agt tct ttg ggc atg gtg aag ttg gag atg   1745
Ala Val Val Phe Asn Asn Ser Ser Leu Gly Met Val Lys Leu Glu Met
        460                 465                 470

ctc gtg gag gga cag cca gaa ttt ggt act gac cat gag gaa gtg aat   1793
Leu Val Glu Gly Gln Pro Glu Phe Gly Thr Asp His Glu Glu Val Asn
    475                 480                 485

ttc gca gag att gcg gcg gct gcg ggt atc aaa tcg gta cgc atc acc   1841
Phe Ala Glu Ile Ala Ala Ala Ala Gly Ile Lys Ser Val Arg Ile Thr
490                 495                 500                 505

gat ccg aag aaa gtt cgc gag cag cta gct gag gca ttg gca tat cct   1889
Asp Pro Lys Lys Val Arg Glu Gln Leu Ala Glu Ala Leu Ala Tyr Pro
                510                 515                 520

gga cct gta ctg atc gat atc gtc acg gat cct aat gcg ctg tcg atc   1937
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Gly Pro Val Leu Ile Asp Ile Val Thr Asp Pro Asn Ala Leu Ser Ile
            525                 530                 535

cca cca acc atc acg tgg gaa cag gtc atg gga ttc agc aag gcg gcc   1985
Pro Pro Thr Ile Thr Trp Glu Gln Val Met Gly Phe Ser Lys Ala Ala
        540                 545                 550

acc cga acc gtc ttt ggt gga gga gta gga gcg atg atc gat ctg gcc   2033
Thr Arg Thr Val Phe Gly Gly Gly Val Gly Ala Met Ile Asp Leu Ala
    555                 560                 565

cgt tcg aac ata agg aat att cct act cca tgatgattga tacacctgct     2083
Arg Ser Asn Ile Arg Asn Ile Pro Thr Pro
570                 575

gttctcattg accgcgagcg cttaactgcc aacatttcca ggatggcagc tcacgccggt 2143

gcccatgaga ttgccct                                                2160


<210> 5
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<212> PRT
<213> Corynebacterium glutamicum

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Met Ala His Ser Tyr Ala Glu Gln Leu Ile Asp Thr Leu Glu Ala Gln
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Gly Val Lys Arg Ile Tyr Gly Leu Val Gly Asp Ser Leu Asn Pro Ile
            20                  25                  30

Val Asp Ala Val Arg Gln Ser Asp Ile Glu Trp Val His Val Arg Asn
        35                  40                  45

Glu Glu Ala Ala Ala Phe Ala Ala Gly Ala Glu Ser Leu Ile Thr Gly
    50                  55                  60

Glu Leu Ala Val Cys Ala Ala Ser Cys Gly Pro Gly Asn Thr His Leu
65                  70                  75                  80

Ile Gln Gly Leu Tyr Asp Ser His Arg Asn Gly Ala Lys Val Leu Ala
                85                  90                  95

Ile Ala Ser His Ile Pro Ser Ala Gln Ile Gly Ser Thr Phe Phe Gln
            100                 105                 110

Glu Thr His Pro Glu Ile Leu Phe Lys Glu Cys Ser Gly Tyr Cys Glu
        115                 120                 125

Met Val Asn Gly Gly Glu Gln Gly Glu Arg Ile Leu His His Ala Ile
    130                 135                 140

Gln Ser Thr Met Ala Gly Lys Gly Val Ser Val Val Val Ile Pro Gly
145                 150                 155                 160

Asp Ile Ala Lys Glu Asp Ala Gly Asp Gly Thr Tyr Ser Asn Ser Thr
                165                 170                 175

Ile Ser Ser Gly Thr Pro Val Val Phe Pro Asp Pro Thr Glu Ala Ala
            180                 185                 190

Ala Leu Val Glu Ala Ile Asn Asn Ala Lys Ser Val Thr Leu Phe Cys
```

195 200 205

Gly Ala Gly Val Lys Asn Ala Arg Ala Gln Val Leu Glu Leu Ala Glu
210 215 220

Lys Ile Lys Ser Pro Ile Gly His Ala Leu Gly Gly Lys Gln Tyr Ile
225 230 235 240

Gln His Glu Asn Pro Phe Glu Val Gly Met Ser Gly Leu Leu Gly Tyr
245 250 255

Gly Ala Cys Val Asp Ala Ser Asn Glu Ala Asp Leu Leu Ile Leu Leu
260 265 270

Gly Thr Asp Phe Pro Tyr Ser Asp Phe Leu Pro Lys Asp Asn Val Ala
275 280 285

Gln Val Asp Ile Asn Gly Ala His Ile Gly Arg Arg Thr Thr Val Lys
290 295 300

Tyr Pro Val Thr Gly Asp Val Ala Ala Thr Ile Glu Asn Ile Leu Pro
305 310 315 320

His Val Lys Glu Lys Thr Asp Arg Ser Phe Leu Asp Arg Met Leu Lys
325 330 335

Ala His Glu Arg Lys Leu Ser Ser Val Val Glu Thr Tyr Thr His Asn
340 345 350

Val Glu Lys His Val Pro Ile His Pro Glu Tyr Val Ala Ser Ile Leu
355 360 365

Asn Glu Leu Ala Asp Lys Asp Ala Val Phe Thr Val Asp Thr Gly Met
370 375 380

Cys Asn Val Trp His Ala Arg Tyr Ile Glu Asn Pro Glu Gly Thr Arg
385 390 395 400

Asp Phe Val Gly Ser Phe Arg His Gly Thr Met Ala Asn Ala Leu Pro
405 410 415

His Ala Ile Gly Ala Gln Ser Val Asp Arg Asn Arg Gln Val Ile Ala
420 425 430

Met Cys Gly Asp Gly Gly Leu Gly Met Leu Leu Gly Glu Leu Leu Thr
435 440 445

Val Lys Leu His Gln Leu Pro Leu Lys Ala Val Val Phe Asn Asn Ser
450 455 460

Ser Leu Gly Met Val Lys Leu Glu Met Leu Val Glu Gly Gln Pro Glu
465 470 475 480

Phe Gly Thr Asp His Glu Glu Val Asn Phe Ala Glu Ile Ala Ala Ala
485 490 495

Ala Gly Ile Lys Ser Val Arg Ile Thr Asp Pro Lys Lys Val Arg Glu
500 505 510

Gln Leu Ala Glu Ala Leu Ala Tyr Pro Gly Pro Val Leu Ile Asp Ile
515 520 525

Val Thr Asp Pro Asn Ala Leu Ser Ile Pro Pro Thr Ile Thr Trp Glu

530 535 540

Gln Val Met Gly Phe Ser Lys Ala Ala Thr Arg Thr Val Phe Gly Gly
545 550 555 560

Gly Val Gly Ala Met Ile Asp Leu Ala Arg Ser Asn Ile Arg Asn Ile
565 570 575

Pro Thr Pro

<210> 6
<211> 875
<212> DNA
<213> Corynebacterium glutamicum

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gcaggtgacg gtacttattc caattccact atttcttctg gcactcctgt ggtgttcccg 180
gatcctactg aggctgcagc gctggtggag gcgattaaca acgctaagtc tgtcactttg 240
ttctgcggtg cgggcgtgaa gaatgctcgc gcgcaggtgt tggagttggc ggagaagatt 300
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gaggtcggca tgtctggcct gcttggttac ggcgcctgcg tggatgcgtc caatgaggcg 420
gatctgctga ttctattggg tacggatttc ccttattctg atttccttcc taaagacaac 480
gttgcccagg tggatatcaa cggtgcgcac attggtcgac gtaccacggt gaagtatccg 540
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gatcgttcct tccttgatcg gatgctcaag gcacacgagc gtaagttgag ctcggtggta 660
gagacgtaca cacataacgt cgagaagcat gtgcctattc accctgaata cgttgcctct 720
attttgaacg agctggcgga taaggatgcg gtgtttactg tggataccgg catgtgcaat 780
gtgtggcatg cgaggtacat cgagaatccg gagggaacgc gcgactttgt gggttcattc 840
cgccacggca cgatggctaa tgcgttgcct catgc 875